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Short communication

5′-Terminal stem-loop of carnation ringspot virus RNA1 is required for the efficient amplification of viral RNAs

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ABSTRACT

Carnation ringspot virus (CRSV) is the prototype virus of the genus *Dianthovirus*. Full-length cDNAs of CRSV strainsPV-0097 and PV-21 were constructed and the infectivity of *in vitro* transcripts was analyzed. Infectivity of PV-0097 and PV-21 to several plants was markedly higher than that of 1.30, a previously reported infectious CRSV clone. Overall RNA sequences of these viruses were similar, but PV-0097 and PV-21 contained additional nucleotides at the 5′ end of RNA1. Stem-loop structures were predicted in the 5′-terminal region of PV-0097 and PV-21 RNA1 but not in 1.30 RNA1. Mutant CRSV 1.30 RNA1 that contains the terminal 4 nucleotides of PV-0097, predicted to fold a 5′-terminal stem-loop structure, recovered higher level accumulation of viral RNAs in the inoculated protoplasts and leaves of *Nicotiana benthamiana*. These results suggest that the 5′-terminal stem-loop structure of CRSV RNA1 plays an important role in efficient amplification of the virus.

The genus *Dianthovirus* in the family *Tombusviridae* is a group of viruses with icosahedral virion and a genome of positive-stranded RNA with no cap structure at the 5′ end, nor poly A tail at the 3′ end. Among eight genera in the family, only the genus *Dianthovirus* contains viruses with a segmented genome structure. The molecular mechanisms of dianthovirus multiplication have been elucidated using mostly red clover necrotic mosaic virus (RCNMV) (Okuno and Hiruki, 2013). Cap- and poly(A)-independent translation of RCNMV replication proteins encoded by genome RNA1 is mediated by cap-independent translation enhancers (CITEs) in the 3′ untranslated region (UTR) (Mizumoto et al., 2003). The translation mechanism of RNA2 differs from that of RNA1. RNA2 uses different translation initiation factors (Tajima et al., 2017), and the translation of RNA2 has a strong link to RNA replication (Hyodo et al., 2017b; Mizumoto et al., 2006). The replication proteins of RCNMV localizes at the endoplasmic reticulum and recruit a variety of host proteins to form viral replication complexes (VRCs) and replicate viral RNAs (Mine and Okuno, 2012; Hyodo and Okuno, 2016; Hyodo et al., 2017a, 2018). The formation of VRCs is also required for the activity of RNA silencing suppression and efficient cell-to-cell movement (Kaido et al., 2011, 2014; Mine et al., 2010; Takeda et al., 2005).

Carnation ringspot virus (CRSV) is the prototype virus of the genus *Dianthovirus*. Although it has caused, and still potentially can cause major economic diseases of carnation crops worldwide in a super-infection with other viruses such as carnation mottle virus, which also belongs to the family *Tombusviridae* (Hiruki, 1987), the molecular mechanisms of multiplication have not yet been intensively studied. The genome of CRSV is composed of 3.8 kb RNA1 (Ryabov et al., 1994) and 1.4 kb RNA2 (Kendall and Lommel, 1992). RNA1 encodes 27K and 88K proteins that are involved in the replication of the virus genome, and a 37K coat protein (CP). RNA2 encodes a 34K movement protein.

The only example of an infectious cDNA clone reported to date is CRSV 1.30 (Sit et al., 2001). *In vitro* transcripts of this cDNA clone (generous gift from Steven A. Lommel) had only mild infectivity to several plants under our experimental conditions (Table 1). We determined the sequence of the clone and found that no stem-loop structure was expected in the 5′ terminus of RNA1 (Fig. 1B) unlike that of RCNMV (Fig. 1B, Sarawaneeyaruk et al., 2009). In this study, we constructed full-length infectious cDNA clones of two CRSV strains, PV-0097 and PV-21, and compared the sequences and the virulence to several plants with those of CRSV 1.30 strain. We discuss the importance of stem-loop structure(s) at the 5′ terminus of genomic RNA1 for CRSV multiplication.

CRSV PV-0097 strain was obtained from the German Resource Center for Biological Material (DSMZ, Braunshweig, Germany) and PV-21 strain was obtained from American Type Culture Collection (ATCC,

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Table 1
Infectivity and virulence of CRSV.

Plants	CRSV strain	Symptoms		Press bolt	
		I	U	I	U
Nicotiana benthamiana	1.30	Nc	Nc	+	+
	PV-0097	Nc	Nc, D	+ +	+ +
	PV21	Nc	Nc, D	+ +	+ +
Vigna unguiculata	1.30	n.d.	−	−	−
	PV-0097	n.d.	Nc	+ +	+ +
	PV21	n.d.	Nc	+ +	+ +
Chenopodium quinoa	1.30	Nc	−	+	−
	PV-0097	Nc1	−	+ +	−
	PV21	Nc1	−	+ +	−
Hordeum vulgare	1.30	−	−	−	−
	PV-0097	−	−	−	−
	PV21	−	−	−	−

I, inoculated leaves; U, uninoculated upper leaves; n.t., not tested symptoms: Nc, necrosis or necrotic lesions; D, dwarfing; −, no visible symptoms observed. n.d., not determined because of Nc induction in mock-inoculated leaves; press blot: −, no signal detected; +, faint signal detected; + +, strong signal detected 1: necrotic spots appeared 1–2 days earlier than those by 1.30.

Manassas, VA, USA). The viruses were purified through repetitive inoculation to *Nicotiana benthamiana* and cowpea (*Vigna unguiculata* cv. California Blackeye) plants. Virions were purified from the systemically infected cowpea leaves (Tremaine and Ronald, 1976), and virus RNA was extracted using phenol/chloroform in the presence of 1.3% sodium dodecyl sulfate. Partial cDNAs were constructed using primers designed based on the published CRSV sequences (RNA1 for NC_003530, and RNA2 for NC_003531). The 5′- and 3′-end sequences were determined by a RACE method using a SMART RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). Each of four 5′-RACE clones of PV-0097 RNA1 contained the same sequence. On the other hand, four 5′-RACE clones of PV-21 RNA1 contained two kinds of sequences with one nucleotide difference. Two clones contained uridine, while the other two clones contained cytidine at the 36th position from the 5′ terminus (data not shown). We chose the clone that contained uridine, because PV-0097 and 1.30 contained uridine at the corresponding position (Fig. 1A). Each of three 3′-RACE clones of PV-0097 RNA1 and two 3′-RACE clones of PV-21 contained the same sequences, respectively. Full-length cDNAs were synthesized by Superscript III (Thermo Fisher Scientific, Waltham, MA, USA) and amplified by KOD-plus (Toyobo, Osaka, Japan) and cloned into pUC18 plasmid (Takara Bio, Otsu, Japan) with T7 promoter at the 5′ end and *Xba* I site at the 3′ end of the RNA1 clone, or *Hin*dIII site at the 3′ end of the RNA2 clone, respectively. Several cDNA clones were constructed and mixtures of RNA1 and RNA2 transcripts from each clone (without cap analog) were inoculated onto *Chenopodium quinoa* and *N. benthamiana* plants. The combination that induced severe symptoms closest to those induced by the purified virion was selected as the cDNA clones. These clones were sequenced and registered in the DDBJ database as LC460998 and LC461176 for CRSV PV-0097 RNA1 and RNA2, and LC461178 and LC461177 for CRSV PV-21 RNA1 and RNA2, respectively. In addition, we determined the sequence of 1.30 clone and registered it in the DDBJ database as LC461179 and LC461180 for RNA1 and RNA2, respectively.

Seven-day-old barley (*Hordeum vulgare* cv. Hinode Hadaka), 4-week-old *N. benthamiana*, 10-day-old cowpea, and 5-week-old *C. quinoa* plants were mechanically inoculated with 1.0 mg/ml *in vitro* transcripts from each CRSV strain and incubated at 17 °C in a chamber with a 16-h photoperiod per day. Infectivity of three strains of CRSV was investigated by press-blotting (Fujisaki et al., 2003) using rabbit antibody raised against 18-amino-acid CP peptide (IPYATTQIVTTSNPPKKG) of CRSV PV-21 strain (Sigma-Aldrich Japan, Tokyo, Japan). No CRSV strains could infect barley plants (Table 1) or protoplasts (data not shown). Both CRSV strains PV-0097 and PV-21 systemically infected *N. benthamiana* and cowpea efficiently with severe necrotic symptoms (Table 1). CRSV 1.30 strain also systemically infected *N. benthamiana*. However, its symptoms were milder than those induced by PV-0097 and PV-21 strains and its detected CP signals were faint (Table 1). CRSV 1.30 did not induce any detectable symptoms in cowpea, and the CP signals were below the limit of detection in both inoculated and upper leaves of cowpea (Table 1). CRSV PV-0097 and PV-21 induced necrotic spots in the inoculated leaves of *C. quinoa* by 4 days post inoculation (dpi), and strong CP signals were detected. On the other hand, the appearance of necrotic spots was delayed by 1–2 days in 1.30-inocuated leaves and the CP signal was very difficult to detect (Table 1). These results show that CRSV PV-0097 and PV-21 are highly virulent strains and 1.30 strain is moderately virulent.

Next, we compared the sequences of three CRSV clones. The nucleotide sequence of RNA1 of 1.30 strain shares 94.2% identity with that of PV-0097 and 95.7% identity with that of PV-21. The nucleotide sequence of RNA2 of 1.30 strain shares 93.2% identity with those of PV-0097 and PV-21. No nucleotide deletion was found in open reading frames of RNA1 and RNA2 (data not shown). Thus, the nucleotide sequences of the three strains of CRSV are highly homologous overall. However, the 5′ terminus of RNA1 was distinctive among virus strains. RNA1s of highly virulent PV-0097 and PV-21 have 4 and 31 additional nucleotides, respectively, compared with that of 1.30 strain (Fig. 1A). Secondary structure prediction by the Mfold program (http://unafold.rna.albany.edu/?q=mfold/rna-folding-form) showed that the 5′ UTR of PV-0097 RNA1 forms one, and that of PV-21 RNA1 forms two 5′-terminal stem-loops, while 1.30 forms a stem-loop 10 nucleotides from the terminus (Fig. 1B). Interestingly, using Mfold, we found that the addition of four nucleotides (AAUA) to the 5′ terminus of 1.30 RNA1 can lead to the formation of a 5′-terminal stem-loop structure similar to that of PV-0097. We constructed a plasmid to transcribe mutant 1.30 RNA1 that has the additional AAUA at the 5′ terminus (Fig. 1B).

To investigate the importance of the 5′-terminal stem-loop structure (s) of RNA1 in the infectivity of CRSV, we compared viral RNA accumulation of the three strains of CRSV and the mutant CRSV (the mutant 1.30 RNA1 plus wild-type 1.30 RNA2) in *N. benthamiana* protoplasts and plants by northern blotting. As expected from the press blot results (Table 1), the accumulation levels of CRSV PV-0097 and PV-21 RNAs were higher than those of 1.30 in protoplasts and in inoculated leaves (Fig. 2A and B, upper and middle panels, lanes 2–4). The mutant 1.30 also accumulated viral RNA to a level comparable to CRSV PV-0097 and PV-21 in both protoplasts and inoculated leaves (Fig. 2A and B, upper and middle panels, lanes 3–5). These results suggest that the 5′-terminal stem-loop structure of RNA1 is required for efficient accumulation of viral RNA, especially that of RNA1. Similarly, the accumulation levels of PV-0097 and PV-21 RNAs were much higher than those of CRSV 1.30 in upper leaves (Fig. 2A and B, bottom panels, lanes 2–4). However, viral RNAs were poorly detected in the upper leaves of plants inoculated with the mutant 1.30, unlike in protoplasts and in inoculated leaves (Fig. 2A and B, bottom panels, lanes 3–5). This interesting result is discussed later.

In contrast to the difference of RNA1 accumulation in protoplasts and inoculated leaves between 1.30 and other strains, RNA2 accumulation only differed slightly, although the accumulation level of 1.30 RNA2 was somewhat lower than that of other strains (Fig. 2A and B, upper and middle panels, lanes 2 and 3). These data suggest that the lower systemic infectivity of 1.30 strain on *N. benthamiana* is mainly due to the lower accumulation of viral RNA1 at the single-cell level. It is interesting that 1.30 RNA2 accumulated to comparable levels to other strains in spite of the lower accumulation of 1.30 RNA1, which provides viral replicase proteins. CRSV (including 1.30 strain) RNA2 might have a sequence that recruits viral replication proteins, such as the Y-shaped RNA structure in the 3′ UTR of RCNMV RNA2 (An et al., 2010), to which the replicase protein directly binds (Iwakawa et al., 2011). Such

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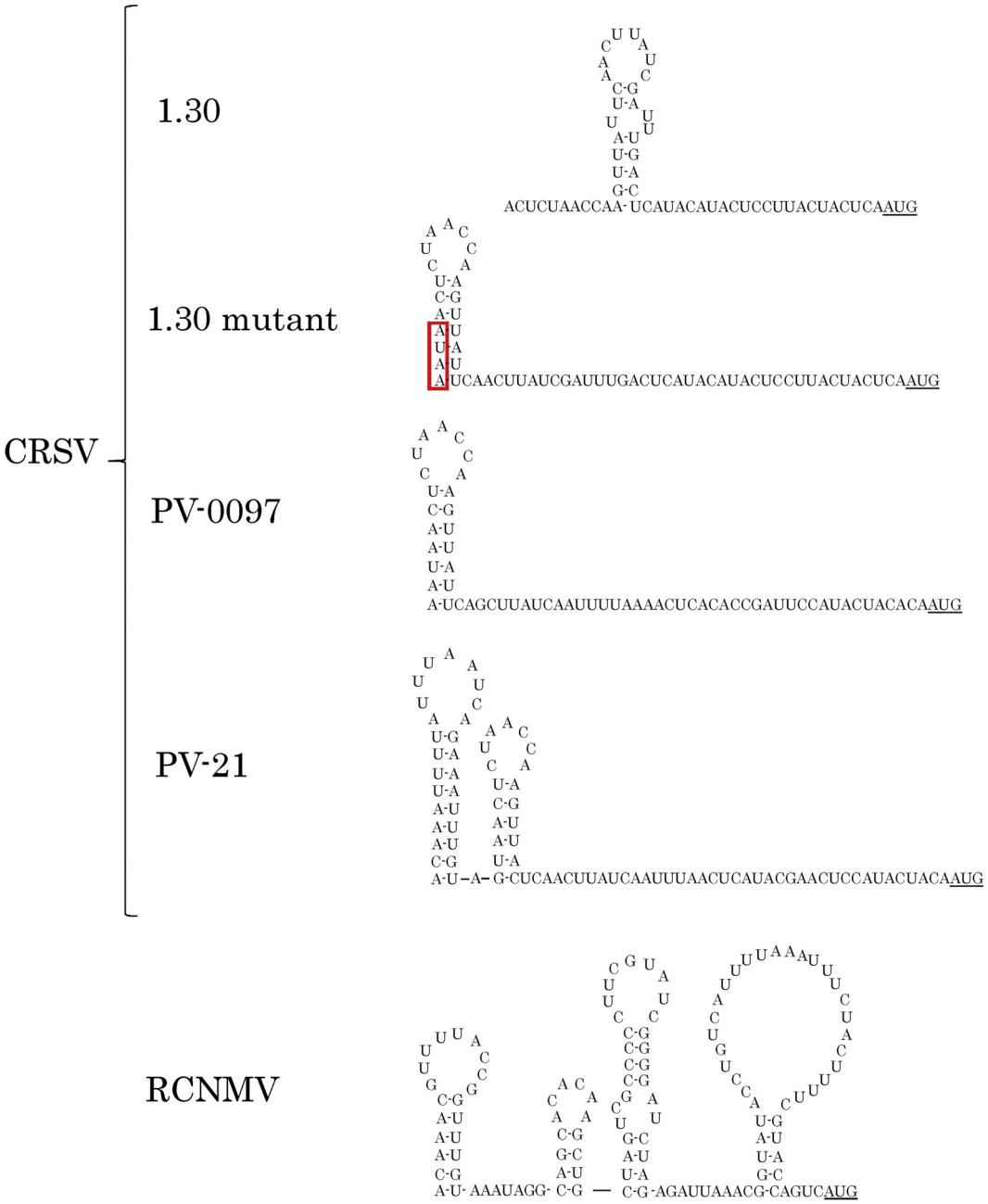

Fig. 1. Nucleotide sequences and secondary structures of the 5′ untranslated region (UTR) of CRSV RNA1. (A) Sequence comparison of RNA1 5′ UTR of CRSV strains 1.30, PV-0097 and PV-21. (B) Predicted secondary structures of three strains of CRSV and a mutant RNA1, and RCNMV RNA1. Red rectangle shows the four nucleotides at the 5′-end of CRSV PV-0097 RNA1. Underlines show the start codon of 27K and 88K proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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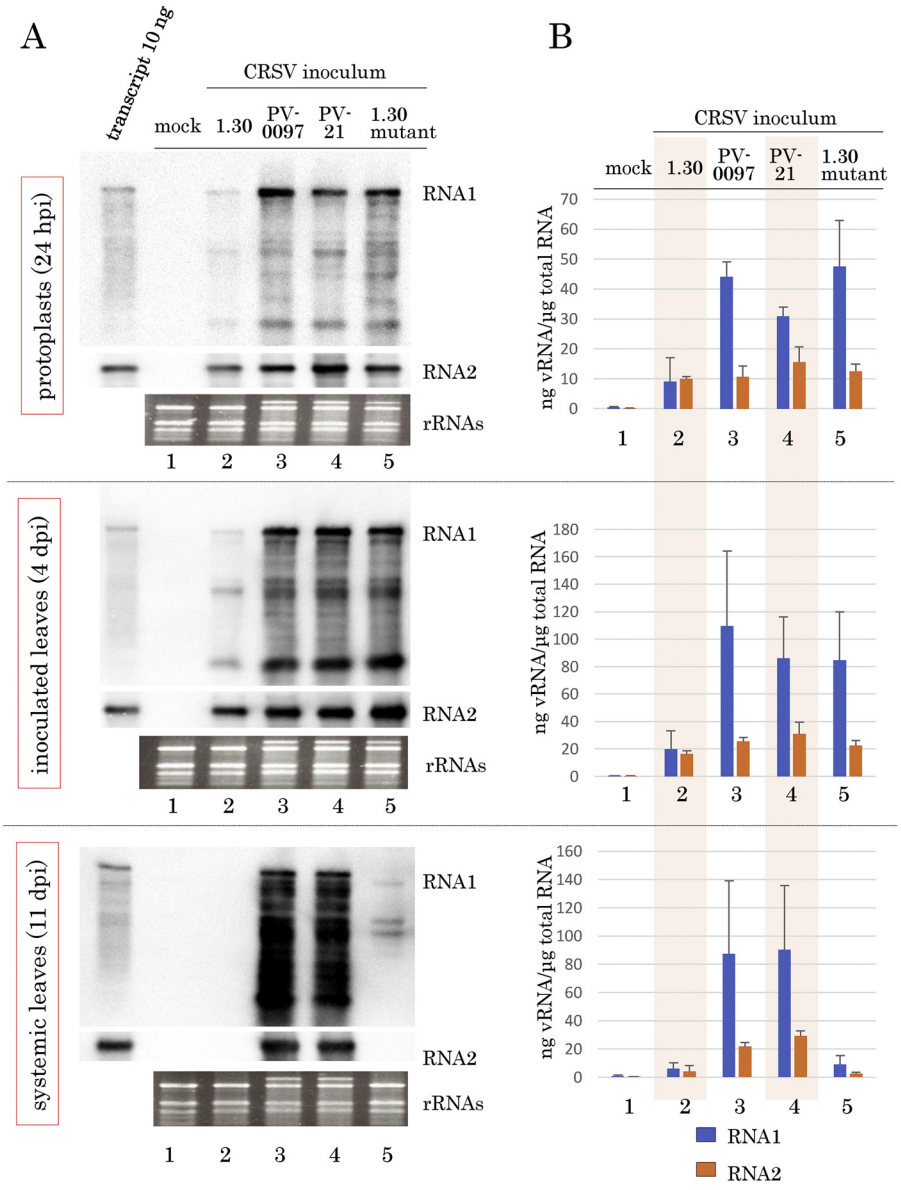

Fig. 2. 5′-Terminal stem-loop is required for efficient amplification of CRSV RNAs in *N. benthamiana*. (A) Accumulation of positive-strand CRSV RNA1 and RNA2 in *N. benthamiana* protoplasts and leaves. Protoplasts (2×10^5) were inoculated with 5 μg of each strain or mutant CRSV *in vitro* transcripts using polyethylene glycol and incubated at 17 °C for 24 h. Four-week-old plants were mechanically inoculated with 5 μg of CRSV *in vitro* transcripts and incubated at 17 °C; the inoculated leaves were sampled at 4 dpi and upper leaves were sampled at 11 dpi. Total RNA was extracted and analyzed by northern blotting using DIG-labeled probes specific for positive-strand RNA1 or RNA2 of CRSV PV-21 strain. The probes can detect the three strains of CRSV RNAs in a similar sensitivity. One microgram of total RNA was loaded to each lane. Ethidium bromide-stained ribosomal RNAs (rRNAs) are shown below the northern blots as the loading control. (B) Relative accumulation of positive-strand CRSV RNAs in *N. benthamiana* protoplasts and leaves. Three independent northern blotting results as shown in (A) were analyzed using ImageJ software.

RNA elements may allow CRSV RNA2 to be replicated efficiently even when the concentration of replicase proteins is low. To reinforce this hypothesis, Mfold predicted a Y-shaped structure in the 3′ UTR of all three strains of CRSV (An et al., 2010, data not shown).

As described above, the 5′-terminal stem-loop structure is important for efficient accumulation of RNA1, and probably for virulence of the virus. At present it is unclear at which stage of viral multiplication the 5′-terminal stem-loop structure is involved. Our previous study has shown that all four stem-loop structures (SL1–SL4) in the 5′ UTR of RCNMV RNA1 are involved in the stability of RNA1, while the terminal SL1 is also involved in cap-independent translation in *Nicotiana tabacum* BY-2 protoplasts, although the association with 3′-CITEs is unknown (Sarawaneeyaruk et al., 2009). All three strains of CRSV are predicted to form secondary structures close to RCNMV 3′-CITEs (Fig. S1A) in the 3′ UTR of RNA1, and one of the stem-loops has the same sequence (Fig. S1A and B). Four nucleotides in the loop are complementary to the sequence in the 5′-terminal stem-loop of PV-0097 and the mutant 1.30 RNA1. Both of the 5′-terminal stem-loops in PV-21 RNA1 have the complementary four nucleotides (Fig. S1B). These data might indicate that the 5′-terminal stem-loop is involved in cap-independent translation through a long-distance interaction with 3′-CITEs. We need to undertake further efforts, such as running RNA stability assays and translation assays using reporter genes such as the luciferase gene (Mizumoto et al., 2003; Sarawaneeyaruk et al., 2009).

Finally, we argue the poor accumulation of the mutant CRSV 1.30 RNAs in the upper leaves as low as that of 1.30 strain (Fig. 2A and B, lanes 2 and 5). Two possibilities are considered for this result. One is that MP of 1.30 strain is inefficient in the long-distance movement of the virus in *N. benthamiana*. This is unlikely, however, because our data suggests that pseudo recombinant CRSV (PV-0097 RNA1 plus 1.30 RNA2) systemically infected *N. benthamiana* and the accumulation level of viral RNAs in the upper leaves was similar to that of CRSV PV-0097 strain (Fig. S3). The other is the differences in virion-forming ability between 1.30 strain and other strains. RCNMV CP contains an N-terminal lysine-rich motif that affects virion formation and systemic spread in *N. benthamiana* (Park et al., 2012). Among 339 amino acids, 15 amino acids of 1.30 CP are different from those of both PV-0097 and PV-21 CP (Fig. S2). In particular, lysine is the fourth amino acid conserved in PV-0097 and PV-21 CP, but it changes to arginine in 1.30 CP. Such a difference in 1.30 CP might result in inefficient virion formation and lead to the lower level of systemic infection. Indeed, any nucleotide differences that could affect virion formation might compromise

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systemic movement of the virus.

Conflict of interests

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.virusres.2019.03.009.

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